1 Smith et al., Long-Duration Space Flight and Bed Rest Effects on Testosterone…
2 **Supplemental Material**
3

4 *LC-MS/MS method*. An Oasis HLB 96-well µElution SPE (Waters Corporation, Milford, MA)
5 plate was conditioned with 0.2 mL methanol followed by 0.2 mL deionized water. For
6 determination of total testosterone, 20 µL of internal standard (stable isotope-labeled testosterone-
7 d3, 25 ng/mL in 50% methanol-water solution) was added, along with 100 µL of 5% H_3PO_4 water
8 solution and 500 µL deionized water, to the 100 µL calibrator, control, and serum samples in a
9 1.5-mL microcentrifuge vial with cap. The samples were vortexed and loaded onto the HLB SPE
10 plate. For determination of bioavailable testosterone, 100 µL cold (4–8°C) saturated ammonium
11 sulfate water solution was added to 100 µL calibrator, control, and serum samples. The samples
12 were vortexed and centrifuged for 5 min at 13,500 RPM (18,779 x *g*). The supernatant was
13 transferred to another 1.5-mL microcentrifuge vial and the following were added: 20 µL internal
14 standard T-d3, 20 µL 5% H_3PO_4 and 500 µL deionized water. The samples were vortexed and
15 loaded onto the HLB SPE plate. The SPE plates were rinsed with 0.2 mL 5% NH_4OH water
16 solution followed by 0.2 mL 50% methanol-water solution . The samples were eluted with 0.1
17 mL 100% methanol to a 0.8-mL 96-well sample collection plate and the plate was covered for
18 LC-MS/MS analysis. Ten microliters of the extracted sample was injected into the Ultra
19 Performance (UP) LC-MS/MS system.
20

21 *LC-MS/MS Conditions*. The chromatographic separation was performed using a Waters Acquity
22 UPLC system equipped with an autosampler, a binary pump manager, and a Waters Acquity
23 UPLC BEHC18 analytical column (50 x 2.1 mm, 1.7-µm particle size) (Waters Corporation,
24 Milford, MA). Mobile Phase A was deionized water (0.05% formic acid) and mobile phase B was
25 acetonitrile (0.05% formic acid). The following gradient elution procedure with a flow rate of 2.5
26 mL/min was used for each injection: 0 min of 50% B and held for 0.25 min; a linear increase to

27 80% B at 0.75 min and then up to 100% B in 0.25 min, held for 0.5 min; and then a linear return
28 to the initial state. The total run time was 2 min. Injection volume was 10 μL. Sample chamber
29 temperature was 4°C and column chamber temperature was 30°C during the analysis. Mass
30 detection and quantification of the total and bioavailable testosterone was carried out with a
31 Quattro Micro™ API mass detector using an electrospray interface (Waters). Positive ions were
32 monitored in the multiple reaction modes (MRM). The respective parent/product ion pair was
33 289.3/109.0 m/z for testosterone and 292.3/109.0 m/z for testosterone-d3. The following
34 parameters for the ion source were used: capillary voltage 3.5 kV, cone 30 V, and collision
35 energy 25 eV. Dwell time was 0.3 sec, source temperature was 120°C, and desolvation
36 temperature was 450°C. Argon was used as the collision gas. The whole UPLC-MS/MS system
37 was controlled by MassLynx 4.1 software (Waters).
38

39 *Estimation of SHBG and Free Testosterone.* Free testosterone and SHBG were calculated based
40 on a mathematical model developed by Vermeulen and colleagues (1). We developed a method
41 from this model to estimate SHBG and FT using concentrations of TT and BT that can be
42 determined by the LC-MS/MS, and an average concentration of albumin in human serum. The
43 equation of testosterone in serum is described as:

Total Testosterone = Free T + Alb-bound T + SHBG-bound T

$$[TT] = [FT] + [AT] + [PT]$$

44 $[AT]/[FT] = \text{constant} = K_A[\text{Alb.}] = K_A\,(43\text{g/L})/69000 = 6.23188 \times 10^{-4} K_A$

45 Where 69000 = molecular weight of albumin, and the 43 g/L is average albumin concentration in
46 human serum.

47 $[AT] = 6.23188 \times 10^{-4} K_A\,[FT]$,

48 $[BT] = [FT] + [AT] = (1 + 6.23188 \times 10^{-4} K_A)\,[FT]$

49 Thus FT concentration can be calculated as:

50 $$[FT] = [BT]/ (1 + 6.23188 \times 10^{-4} K_A) \quad (1)$$

51 If [P] = free SHBG, and [PT] = testosterone bound SHBG, we can get an equation as

52 $$[FT] + [P] \leftrightarrow [PT] \text{ or } [P] = [PT]/ ([FT] K_S)$$

53 $$[SHBG] = [P] + [PT] = [PT]/ ([FT] [K_S]) + [PT] = [PT] \{1/ ([FT] K_S) + 1\}$$

54 Where [PT] = [TT] – [BT], and use equation (1) $[FT] = [BT]/ (1 + 6.23188 \times 10^{-4} K_A)$

55 Thus SHBG concentration can be calculated as:

56 $$[SHBG] = ([TT] - [BT])\{(1+6.23188 \times 10^{-4} K_A)/ (K_S[BT]) + 1\} \quad (2)$$

57

58 We calculated SHBG concentrations in 6 serum samples in the College of American Pathologists
59 (CAP) 2010 inter-laboratory survey using concentrations of TT and BT that determined by LC-
60 MS/MS, and optimal association constants $K_S = 2.5 \times 10^9$ L/mol and $K_A = 2.45 \times 10^4$ L/mol (2, 3).
61 Compared to the mean values of SHBG concentrations in the CAP report, our estimated SHBG
62 results showed an average 10.3% deviation. Four of the 6 samples had deviations <10%.

63

64 *Accuracy and Precision of the LC-MS/MS method*. Three levels (75, 300, and 1500 ng/dL) of
65 controls of testosterone were used for the evaluation of intra-assay and inter-assay % CVs. The
66 controls were prepared with spiking testosterone in a 1% BSA water solution. The intra-assay
67 CVs (n=6) were 8.7%, 3.5%, and 1.7%, and inter-assay CVs (n=18) were 7.1%, 9.7%, and
68 10.9%, respectively. The linear range of the calibration curve for testosterone using this method
69 was 20–5000 ng/dL (0.69–173.35 nmol/L). The lower limit of quantification (LLOQ) was 20

70 ng/dL (0.69 nmol/L). We also analyzed 6 serum samples using mass spectrometry in a CAP 2010
71 inter-laboratory survey. Compared to the values of total testosterone in the CAP report (*n*=6), our
72 study results showed an average 10.9% deviation. Four of the 6 samples had deviations <10%.
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